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PYY(3-36) and exendin-4 reduce food intake and activate neuronal circuits in a synergistic manner in mice

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ABSTRACT

Peptide YY(3-36) ((PYY(3-36)) and glucagon like peptide 1 (GLP-1) in combination reduce food intake and body weight in an additive or synergistic manner in animal models and in humans. Nevertheless, the mechanisms behind are not completely understood. The present study aims to investigate the effect of combining PYY(3-36) and the GLP-1 receptor agonist exendin-4 (Ex4) by examining acute food intake and global neuronal activation as measured by c-fos in C57BL/6 J mice. An additive reduction in food intake was found 1.5 h after s.c dosing with the combination of PYY(3-36) (200 µg/kg) and Ex4 (2.5 µg/kg). This was associated with a synergistic enhancement of c-fos reactivity in central amygdalar nucleus (CeA), rostral part of the mediobasal arcuate nucleus (ARH), supratrigeminal nucleus (SUT), lateral parabrachial nucleus (PB), area postrema (AP) and nucleus tractus solitarius (NTS) compared to vehicle, PYY(3-36) and Ex4 individually dosed mice. The regions activated by Ex4 individually and PYY(3-36) and Ex4 in combination resembled each other, but the combination group had a significantly stronger c-fos response. Twenty-five brain areas were activated by PYY(3-36) and Ex4 in combination compared to vehicle versus nine brain areas by Ex4 individually. No significant increase in c-fos reactivity was found by PYY(3-36) compared to vehicle dosed mice. The neuronal activation of ARH and the AP/NTS to PB to CeA pathway is important for appetite regulation while SUT has not previously been reported in the regulation of energy balance. As PYY(3-36) and Ex4 act on different neurons leading to recruitment of different signalling pathways within and to the brain, an interaction of these pathways may contribute to their additive/synergistic action. Thus, PYY(3-36) boosts the effect of Ex4 possibly by inducing less inhibition of neuronal activity leading to an enhanced neuronal activity induced by Ex4.

Abbreviation: ACB, Nucleus accumbens; AgRP, Agouti-related peptide; AOB, Accessory olfactory bulb; AP, Area postrema; ARH, Arcuate hypothalamic nucleus; AUDd, Dorsal auditory area; BLA, Basolateral amygdalar nucleus; BMA, Basomedial amygdalar nucleus; BST, Bed nucleus of striatum; CA1, Field CA1; CA2, Field CA2; CA3, Field CA3; CART, Cocaine- and amphetamine-regulated transcript; CeA, Central amygdalar nucleus; COA, Cortical amygdalar area; CU, Cuneate nucleus; DG, Dentate gyrus; DMH, Dorsomedial nucleus of the hypothalamus; DMX, Dorsal motor nucleus of the vagus nerve; DR, Dorsal nucleus raphe; ECT, Ectorhinal area; ECU, External cuneate nucleus; Ex4, Exendin-4; GENd, Geniculate group, dorsal thalamus; GLP-1, Glucagon like peptide 1; GLP-1R, Glucagon like peptide 1 receptor; IC, Inferior colliculus; LC, Locus ceruleus; LH, Lateral habenula; LHA, Lateral hypothalamis area; LPO, Lateral preoptic area; LRNm, Lateral reticular nucleus, magnocellular part; LS, Lateral septal nucleus; ME, Median eminence; MEPO, Median preoptic nucleus; MH, Medial habenula; MM, Medial mammillary nucleus; MPT, Medial pretectal area; MRN, Midbrain reticular nucleus; NI, Nucleus incertus; NLL, Nucleus of the lateral lemnuscus; NPY, Neuropeptide Y; NTS, Nucleus of the solitary tract; OV, Vascular organ of the lamina terminalis; PAG, Periaqueductal gray; PB, Parabrachial nucleus; PCG, Pontine central gray; PG, Pontine gray; PH, Posterior hypothalamic nucleus; POMC, Pro-opiomelanocortin; PSTN, Parasubthalamic nucleus; PVT, Paraventricular thalamus; PVH, Periventricular hypothalamic nucleus; PVp, Periventricular hypothalamic nucleus, posterior part; PYY(3-36), Peptide YY(3-36); RHP, Retrohippocampal region; SCH, Suprachiasmatic nucleus; SCm, Superior colliculus, motor related; SEM, Standard errors of mean; SF, septofimbrial nucleus; SFO, Subfornical organ; SNc, Substantia nigra, compart part; SNr, Substantia nigra, reticular part; SUT, Supratrigeminal nucleus; TTd, Tenia tecta, dorsal part; TU, Tuberal nucleus; Veh, Vehicle; VL, Lateral ventricle; VMH, Ventromedial hypothalamic nucleus; VTA, Ventral tregmental area; V4, Fourth ventricle; ZI, Zona inserta; Y_2, NPY subtype 2 receptor

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1. Introduction

PYY and GLP-1 are gut hormones co-secreted from intestinal L-cells into the circulatory system following a meal to mediate post-prandial satiety. In humans, studies with PYY(3-36) and GLP-1 analogues in combination have shown additive or synergistic effect on food intake (Neary et al., 2005; De Silva et al., 2011; Schmidt et al., 2014). In rodent models, treatment with PYY(3-36) and GLP-1 analogues in combination has shown promising synergistic effects on blood glucose regulation, food intake and body weight reduction (Talsania et al., 2005; Fenske et al., 2012; Reidelberger et al., 2011). To date, no studies have provided evidence of the underlying mechanism of the synergism between PYY(3-36) and GLP-1 on the effect on food intake and body weight reduction. The energy homeostasis is tightly regulated by the central nervous system that integrates signals from the peripheral and central brain regions (Schwartz, 2006; Huo et al., 2009; Sohn et al., 2013). PYY(3-36) has a high affinity for NPY subtype 2 receptors (Y_2) and GLP-1 effectively binds to the GLP-1 receptor (GLP-1R), and both receptors are widely expressed in the vagus nerve (Koda et al., 2005; Ritter, 2004; Nakagawa et al., 2004; Egerod et al., 2018) and the brain (Jensen et al., 2018; Broberger et al., 1997; Stanic et al., 2006; Parker & Herzog, 1999). It is likely that the synergistic effect of PYY(3-36) and GLP-1 is mediated through vagus nerve and appetite-regulating neurons in hypothalamus and hindbrain interacting with each other to supress food intake. The present study investigate the additive/synergistic effect of PYY(3-36) and Ex4 by examining the acute c-fos signal in brain areas in lean C57BL/6J mice. c-fos is a neuronal activity marker giving information of activated brain areas within 30–60 min after treatment (Morgan & Curran, 1991). Identifying and understanding the mechanisms leading to the additive/synergistic effect of PYY(3-36) and GLP-1 could potentially contribute to a treatment with increased efficacy in patients with obesity and diabetes.

2. Methodology

2.1. Experimental animals

The experimental procedures in the present study were approved by the Animal Experiments Inspectorate (2005/561-989), under Danish Ministry of Justice, and are in accordance with the Danish Animal Experimentations Act. The study was performed at the Laboratory Animal Unit at Novo Nordisk A/S, Måløv, Denmark.

2.2. Study design

The present paper included two mice studies. Study I aimed to investigate the acute additive/synergistic effect of PYY(3-36) and Ex4 on food intake and global c-fos reactivity. Study II aimed to investigate the dose-response of Ex4. C57BL/6 J male mice were obtained from Taconic (Denmark). At arrival the mice were single housed in a reversed 12 h/12 h daily rhythm with lights off at 11 am. The mice were fed ad libitum a standard diet with 10% kcal fat (Research Diets #D12450B, New Brunswick NJ, USA) and had free access to water. Prior to study start, the mice were randomised into groups according to body weight to minimize statistical variations between the groups. To habituate and reduce the stress levels that are connected to acute s.c. injection, the mice were handled mimicking injection and weighed daily for 7 days prior to injection. The handling was performed by pinching the mice in the neck between thumb and forefinger. For all experiments, food was removed 3 h prior to dark phase and given back at the onset of dark. The mice were dosed s.c. 45 min before onset of dark and received two injections per experiment (n = 8). Injection volume per compound was 2.2 ml/kg. The compounds were made at Novo Nordisk A/S (Måløv, Denmark). PYY(3-36) was dissolved in 5 mM acetate, 250 nM glycerol, 0.05% tween 80, pH 4.0 and Ex4 was dissolved in 50 mM phosphate, 70 nM NaCl, 0.05% tween 80, pH 7.4. For study I, 32 mice aged 33 weeks and weighing 29-39 g were included (n = 8/group). For both the acute food intake and global brain c-fos reactivity study, the vehicle dosed mice were dosed with vehicle buffers for both PYY(3-36) and Ex4, mice in the PYY(3-36) group were dosed with PYY(3-36) (200 μg/kg) and vehicle buffer for Ex4, mice in Ex4 group were dosed with Ex4 (2.5 μg/kg) and vehicle buffer for PYY(3-36) and mice in the combination group were dosed with PYY(3-36) (200 μg/kg) and Ex4 (2.5 μg/kg). For study II, 24 mice aged 10 weeks and weighing 24-29 g were included (n = 8/group). The used doses for dose-response of Ex4 were 2.5 μg/kg, 90 μg/kg, 170 μg/kg. The vehicle dosed mice received vehicle buffers for both PYY(3-36) and Ex4, while the Ex4 dosed mice received vehicle buffer for PYY(3-36).

2.3. Biodaq system for food intake measurement

Food intake was measured using a BioDaq system (Research Diets, Inc. New Brunswick NJ, USA). The mice were acclimated for 7 days in the BioDAQ system before acute food intake measurements in response to the treatments. On the experimental day, food was removed from the mice 3 h prior to dark phase and given back at the onset of dark. The mice were s.c. injected 45 min before onset of dark, and the access of food was available at the onset of dark. The mice received two single s.c. injections; either veh-PYY(3-36/veh-Ex4, PYY(3-36/veh-Ex4, Ex4/veh-PYY(3-36) or PYY(3-36)/Ex4 (2.2 ml/kg). For the dose-response of Ex4, the mice were also received two single s.c injections of either veh-PYY(3-36)/veh-Ex4 or Ex4/veh-PYY(3-36) in the different doses. Food intake was measured during 22 h.

2.4. Global brain c-fos

The mice were handled mimicking injection and weighed daily for 11 days prior to injection. On the experimental day, food was removed from the mice 3 h prior to dark phase and given back at the onset of dark. The mice received two single s.c. injections 45 min before onset of dark phase; either veh-PYY(3-36)/veh-Ex4, PYY(3-36)/veh-4, Ex4/veh-PYY(3-36) or PYY(3-36)/Ex4 (2.2 ml/kg). 1.5 h after dosing, the mice were anesthetized with isofluran followed by cardiac perfusion under anaesthesia. The mice were perfused (30 ml/min) with saline followed by 4% paraformaldehyde (PFA). The brains were dissected and stored in 4% PFA overnight at room temperature and transferred stepwise to 100% methanol in ddH2O (20% methanol for 1 h, 40% methanol for 1 h, 60% methanol for 1 h, 80% methanol for 1 h, and 100% methanol for 1 h twice). For immunostaining with c-fos the iDISCO was performed as described in (Salinas et al., 2018) with one exception: The primary antibody was Rabbit-a-cfos (1:5000, cat. # 2250, Cell Signalling), and incubation times for primary and secondary antibody were extended to 7 days each.

2.5. Quantification of c-fos reactivity

A cell segmentation algorithm was used prior to construction of c-fos brain reactivity heat maps, which were then used for visualization and quantification. Quantification was performed by summation of the heat map intensity value of all voxels within the individual brain regions, indicating the total brain activation signal in these regions. The results were reported as fold change compared to the vehicle treated group (further details, see (Jensen et al., 2018)). For details of brain atlas and image registration, see reference (Jensen et al., 2018). However, the c-fos intensity for BST, CeA, rostral part of the mediobasal ARH and compact DMH were calculated in defined images in Allen brain atlas. BST; image 49-52 (corresponding to bregma 0.50 to 0.14/0.02 in Paxinos and Franklin's mouse brain atlas). CeA; image 68-74 (bregma −1.34 to −2.06). Rostral part of the mediobasal ARH; image 68-72 (bregma −1.58 to −1.70). Compact DMH; image 72–74 (bregma −1.82 to −2.06).

2.6. Statistics

Data obtained from the study were analyzed and associated graphs were created in GraphPad Prism version 7.00 (GraphPad Software Inc., La Jolla, CA, USA). The statistical analysis of acute food intake and selected c-fos brain areas (Supplemental Fig. 1) were performed by a two sided ANOVA followed by a Tukeys's multiple comparisons test to test for significance between groups. c-fos heat map intensities were analyzed using multiple *t*-tests assuming unequal variance. Statistical significance was determined by correcting for multiple comparisons by a false discovery rate set to 10%. Total c-fos intensity is presented as fold change relative to vehicle treated groups. Total c-fos intensity (Supplemental Fig. 1) and acute food intake at 1.5 h after dosing are presented as means ± standard errors of mean (SEM). A p-value of 0.05 or less was considered statistically significant.

3. Results

3.1. Acute food intake and body weight change

The accumulated food intake over 22 h showed that PYY(3-36) (200 μg/kg) and Ex4 (2.5 μg/kg) in combination resulted in reduced food intake compared to vehicle and PYY(3-36) dosed mice until 10 h after dosing (Fig. 1A). Mice treated with PYY(3-36) (200 μg/kg) only reduced food intake until 2 h after dosing compared to vehicle, while Ex4 (2.5 μg/kg) dosing reduced food intake compared to vehicle dosed mice until 8 h after dosing (Fig. 1A). At 1.5 h after dosing, PYY(3-36) and Ex4 in combination reduced food intake with approximately 100% compared to vehicle ($p < .0001$), and also significant reduced compared to PYY(3-36) ($p < .05$) and Ex4 ($p < .01$). When subjected to statistical evaluation of synergism, there was no statistical interaction/synergistic effect between PYY(3-36) and Ex4. A single administration of either PYY(3-36) and Ex4 resulted in almost 50% reduction in food intake compared to vehicle ($p < .05$, $p < .01$) (Fig. 1B). 24 h after dosing, the mice treated with PYY(3-36) and Ex4 in combination had the greatest change in body weight compared to vehicle and PYY(3-36) treated mice ($p < .001$), while no differences were observed compared to Ex4 (Fig. 1C). Based on the effects on food intake at 1.5 h, it was decided to investigate the brain c-fos reactivity 1.5 h after dosing. Dose response of Ex4 was also tested to investigate if a higher dose of Ex4 could lower food intake to same extent as with the combination of PYY (3-36) and Ex4, and this resulted in a dose-dependent reduction in food intake ($p < .05$, $p < .001$) (Fig. 1D). However, the doses of Ex4 did not lower food intake to the same extent as with PYY(3-36) and Ex4 in combination ($p < .001$, $p < .05$) (Fig. 1B & D).

3.2. Acute global brain c-fos reactivity

Mice treated with PYY(3-36) showed no significant increase in c-fos immunoreactivity compared to the vehicle treated mice (Supplemental Fig. 1A & Fig. 3), while mice treated with Ex4 had enhanced c-fos reactivity in 9 brain regions compared to vehicle including central amygdalar nucleus (CeA), the lateral hypothalamic area (LHA), the parasubthalamic nucleus (PSTN), the paraventricular thalamus (PVT), locus ceruleus (LC), area postrema (AP), cuneate nucleus (CU), dorsal motor nucleus of the vagus nerve (DMX) and the nucleus of the solitary tract (NTS) (Supplemental Fig. 1B & Fig. 3). When combining PYY(3-36) and Ex4, a total of 25 brain regions were significantly upregulated in c-fos immunoreactivity compared to vehicle treated mice. The activation of c-fos was evident in the same regions as with Ex4 individually, but to a significantly greater extend and additional 16 regions were positive for c-fos in the combination group. The additional 16 brain regions included the bed nucleus of striatum (BST), arcuate hypothalamus nucleus (ARH), the dorsomedial nucleus of the hypothalamus (DMH), lateral preoptic area (LPO), median eminence (ME), medial mammilary nucleus (MM), periventricular hypothalamic nucleus, posterior part (PVp), tuberal nucleus (TU), ventromedial hypothalamic nucleus (VMH), lateral habenula (LH), ventral tregmental area (VTA), parabrachial nucleus (PB), pontine central gray (PCG), supratrigeminal nucleus (SUT), and the lateral reticular nucleus, magnocellular part (LRNm) (Supplemental Fig. 1C & Fig. 3). Within CeA, rostral mediobasal ARH, PB, SUT, AP, and NTS an interaction between PYY(3-36) and Ex4 was furthermore responsible for the c-fos signal, suggesting a synergistic effect in neuronal activation. The c-fos signal intensities in each brain region were compared between the group receiving the PYY (3-36) and Ex4 in combination and the group receiving Ex4, to illustrate the differences in neuronal activation between those two groups (Fig. 2). Seven regions in the PYY(3-36) and Ex4 group displayed a significantly increase in c-fos response compared to Ex4 individually namely the CeA, rostral part of the mediobasal ARH, SUT, PB, AP, NTS and LRNm (Figs. 2 and 3). In Supplemental Fig. 2, examples of comparing images of c-fos nucleus with heat map of c-fos intensity in PB and AP/NTS for the different groups are illustrated.

4. Discussion

The present study demonstrated global activation of c-fos in the brain following administration of PYY(3-36) and Ex4 in combination.

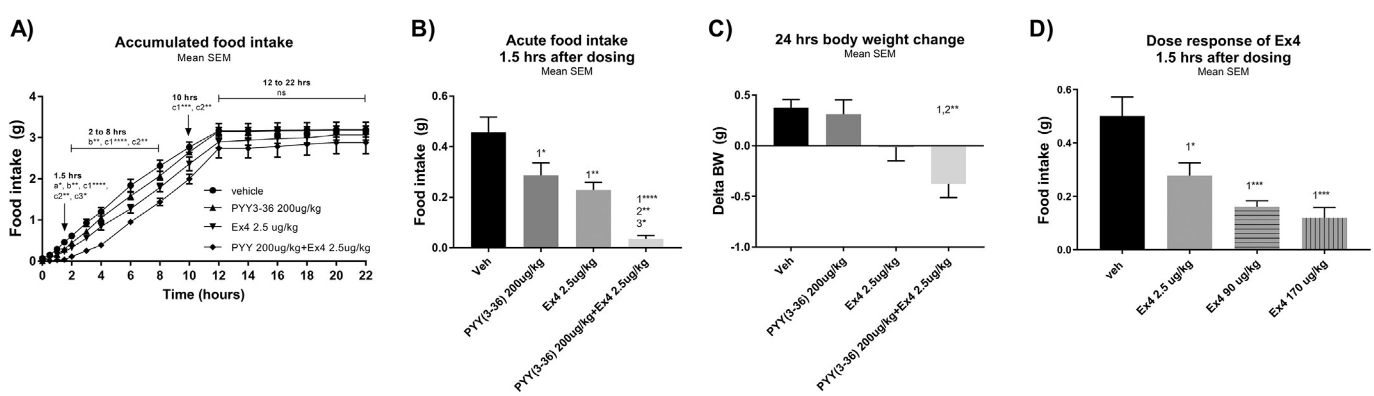

Fig. 1. Food intake and body weight change after a single s.c. injection of PYY(3-36) and Ex4 and the combination thereof: A) Acute accumulated food intake (0 – 22 h), B) Acute food intake 1.5 h after dosing, C) 24 h body weight change after dosing, D) Dose response of Ex4 on food intake 1.5 h after dosing. Data are expressed as means ± SEM, $n = 6–8$, analyzed by two sided ANOVA followed by a Tukeys's multiple comparisons test to test for significance between groups. In Fig. 1A, "a" indicates significant differences between vehicle and PYY(3-36), "b" significant differences between vehicle and Ex4, "c1" significant differences between vehicle and PYY(3-36) and Ex4 combination, "c2" significant differences between PYY(3-36) and PYY(3-36) and Ex4 combination, "c3" significant differences between Ex4 and PYY(3-36) and Ex4. In Fig. 1B–D, "1" indicates significant differences to the vehicle, "2" significant differences to PYY(3-36), "3" significant differences to Ex4. ($p < .05$*, $p < .01$**, $p < .001$***).

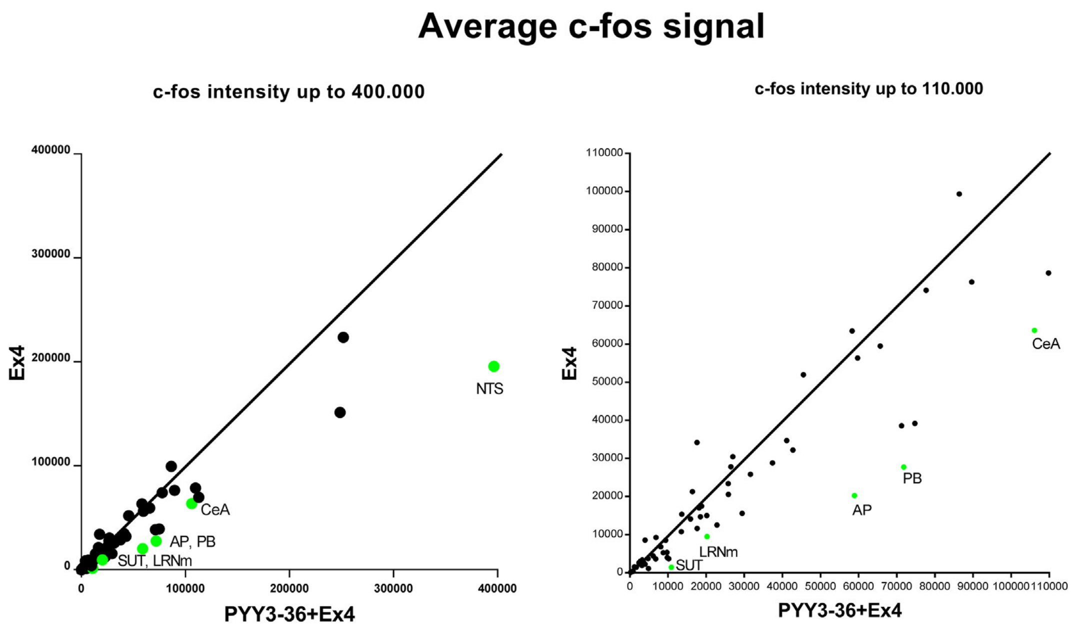

Fig. 2. Comparison of average c-fos signal intensities in each brain region between the PYY(3-36) and Ex4 in combination and the Ex4 group. Scatter plot of summed heat map signal in brain regions with Ex4 values (y-axis) plotted against PYY3-36 + Ex4 (x-axis). The graph to the right is a close-up of the graph to the left. Only regions that were significant different between PYY(3-36) and Ex4 and Ex4 individually are marked. The regions that displayed a significantly increase in c-fos response in the PYY(3-36) and Ex4 group compared to Ex4 individually were CeA, rostral part of the mediobasal ARH, SUT, PB, AP, NTS and LRNm (see also Supplemental Fig. 1). Data, n = 6–8, are analyzed by two sided ANOVA followed by a Tukeys's multiple comparisons test to test for significance between groups.

We show that the additive reduction in food intake 1.5 h after co-dosing with PYY(3-36) and Ex4 is associated with synergistic enhanced c-fos reactivity in CeA, rostral part of the mediobasal ARH, SUT, PB, AP, NTS, and LRNm compared to vehicle, PYY(3-36) and Ex4 dosed mice. Many neurons express the immediate-early gene c-fos when activated and this protein has been considered as a robust marker to study neuronal activity (Morgan & Curran, 1991). However, c-fos is not a global marker and we are aware that not all neuronal activity was captured. Hence, there may be additional differences in brain activation and inhibition which were not captured by this method. To ensure an acute additive/synergistic effect of the PYY(3-36) and Ex4, acute food intake was measured prior to the c-fos analysis. The brain regions activated by PYY(3-36) and Ex4 in combination resembled the regions activated by Ex4 individually, but with a significantly stronger c-fos response and with more regions significantly affected in the combination group (Supplemental Fig. 1). PYY(3-36) has been demonstrated to have an inhibitory effect on neurons (Batterham et al., 2002; Riediger et al., 2004) and no remarkable increase in c-fos reactivity was observed with PYY(3-36) alone compared to vehicle dosed mice. Thus, the synergistic activation of c-fos, indicates that PYY(3-36) in combination with Ex4 boosts the effect of Ex4, which could be through input from vagus nerve and disinhibition of Ex4 induced neuronal activity.

It could be speculated whether the observed c-fos reactivity was induced by fasting as the mice dosed with PYY(3-36) and Ex4 in combination only consumed 0,04 g of food which nearly resemble a fasting condition. However, in a previous study, fasted mice (fasted during light phase as in the present study), no c-fos reactivity was observed in AP and NTS (Riediger et al., 2004) in contrast to the present study results. In addition, mice dosed with either PYY(3-36) or Ex4 individually, consumed approximately the same amount of food and the c-fos reactivity in these two groups was also different suggesting an interaction of the combination treatment rather that fasting. Furthermore, the massive suppression in food intake appears to be specific for PYY(3-36) and Ex4 in combination as a 68 times higher dose of Ex4 (170 μg/kg) alone compared to the Ex4 (2.5 μg/kg) dose could not lower the appetite to the same extent (Fig. 1B & D). This could be interpreted as follows: the high Ex4 dose alone is not sufficient to lower food intake but needs additional activation of non GLP-1R neuron populations to further lower the food intake. The high Ex4 dose (170 μg/kg) may induce a counter-regulatory response to avoid a marked reduction in appetite, and PYY(3-36) may ameliorate this effect potentially through disinhibition of the same areas. However, as no prior scientific evidence for a counter-regulatory response has been shown, this remains speculative. The enhanced c-fos reactivity could also be induced by several additional mechanisms such as 1) an increased access of PYY(3-36) and Ex4 into brain (Salinas et al., 2018; Kastin & Akerstrom, 2003; Kastin et al., 2002; Nonaka et al., 2003), 2) increased interaction of appetite-regulating neurons and cross talk between different regions of the brain (Sohn et al., 2013; Carter et al., 2013), and 3) increased input from vagus nerve (Koda et al., 2005; Egerod et al., 2018; Iwasaki et al., 2013; Kakei et al., 2002). Hence, recruitment of different signalling pathways within and to the brain by PYY(3-36) and Ex4 may contribute to their additive/synergistic action. It is well demonstrated that the anorexigenic effect of PYY(3-36) is mediated both through activating the vagal afferent (Koda et al., 2005; Iwasaki et al., 2013) and through inhibition of the orexigenic AgRP/NPY neurons in ARH (Batterham & Bloom, 2003; Shi et al., 2013). AgRP/NPY neurons co-express GABA which antagonizes several neurons such as the CART/POMC neurons in the ARH (Secher et al., 2014), the CGRP neurons in the PB (Wu et al., 2009), neurons in the BST (Betley et al., 2013) and PVH neurons via Y_1 receptors and AgRP inhibiting the MC4R (Cone, 2005). For GLP-1, the acute anorectic effect is also mediated through activation of the vagal afferent (Kakei et al., 2002) and in ARH through activation of POMC/CART neurons and a simultaneous indirect GABAergic inhibition of the AgRP/NPY neurons (Secher et al., 2014; Barsh & Schwartz, 2002). The enhanced c-fos in the rostral part of the mediobasal ARH (and PVp) was interesting as it contains a large population of AgRP/NPY and POMC/CART neurons (Broberger et al., 1997). Increased POMC/CART activity due to reduced inhibition from AgRP/NPY neurons together with GLP-1R binding of Ex4 on the POMC/CART neurons may reflect the increased c-fos in ARH and PVp (Batterham et al., 2002; Challis et al., 2003). Some research groups have observed increased c-fos reactivity with PYY(3-36) individually in different brain regions such as BST, CeA, ARH, PB, AP, NTS (Koda et al., 2005;

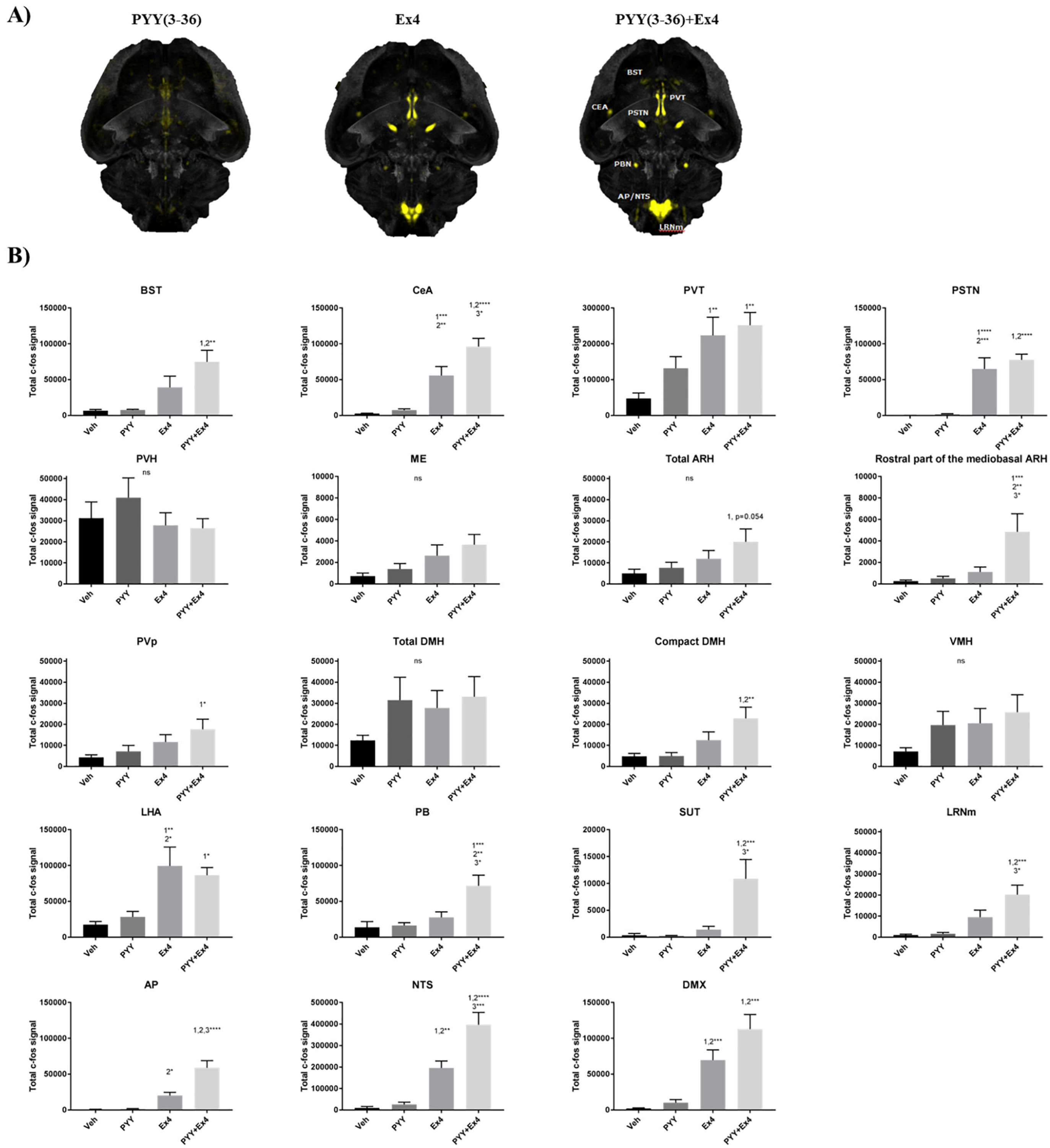

Fig. 3. Total c-fos signal in selected brain regions (selected from comparison of average c-fos signal intensities (Fig. 2) and heat maps (Supplemental Fig. 1)). Note the differences in y-axes. For quantification of c-fos reactivity, a cell segmentation algorithm was used prior to construction of c-fos brain reactivity heat maps, which were then used for visualization and quantification (for further details, see methodology section). Bed nucleus of striatum (BST), central amygdalar nucleus (CeA), paraventricular thalamus (PVT), parasubthalamic nucleus (PSTN), periventricular hypothalamus (PVH), median eminence (ME), arcuate hypothalamus nucleus (ARH), periventricular hypothalamic nucleus, posterior part (PVp), the dorsomedial nucleus of the hypothalamus (DMH), ventromedial hypothalamic nucleus (VMH), the lateral hypothalamic area (LHA), parabrachial nucleus (PB), supratrigeminal nucleus (SUT), lateral reticular nucleus, magnocellular part (LRNm) area postrema (AP), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus nerve (DMX). "1" indicates significant differences to the vehicle, "2" significant differences to PYY(3-36), "3" significant differences to Ex4. ($p < .05^*$, $p < .01^{**}$, $p < .001^{***}$).

Batterham et al., 2002; Baraboi et al., 2010; Halatchev et al., 2004). This could be related to a higher dose of PYY(3-36) and thus indirect c-fos induction as shown with POMC because of less GABAergic inhibition from NPY/AgRP neurons (Batterham et al., 2002; Challis et al., 2003). Moreover, NPY neurons are widely expressed in both hypothalamus and hindbrain, and a large population of these may also express the Y_2 receptor as shown for ARH (Broberger et al., 1997). Thus, NPY or Y_2 positive neurons in several brain regions could be inhibited by direct PYY(3-36) binding of Y_2 allowing for additional GLP-1R signalling to occur in secondary brain regions.

Furthermore, the hindbrain showed strong neuronal activity. Recently, Egerod and colleagues demonstrated that GLP-1R and Y_2 were expressed in vagal afferents, both co-expressed with cholecystokinin receptor 1 (CCK1R) which may also be important for the synergy of PYY(3-36) and Ex4 (Egerod et al., 2018) and the observed synergistic c-fos reactivity in the hindbrain. For appetite reduction, the AP/NTS to PB signalling pathway has been suggested with several appetite reducing compounds such as GLP-1 analogues (Salinas et al., 2018; Swick et al., 2015; Baraboi et al., 2011; Rowland et al., 1997), amylin (Rowland et al., 1997; Lutz, 2005), CCK-8 (Li & Rowland, 1995) and in mice with RYGB (Mumphrey et al., 2016). The PB receives and integrates neuronal input from various brain regions, especially the hypothalamus and hindbrain (Carter et al., 2013; Tokita et al., 2009; Papas & Ferguson, 1990). For control of appetite and blood glucose, neurons in the PB project to CeA and BST (Carter et al., 2013; Meek et al., 2016; Campos et al., 2016). In the present study, two clusters of significantly increased c-fos reactivity were found in the PB for PYY(3-36) and Ex4 in combination which were not observed for PYY(3-36) or Ex4 individually. CGRP neuron expression is characteristic to PB and mediates appetite reduction through innervation of CeA (Mumphrey et al., 2016). In the RYGB mice c-fos reactivity was co-expressed with CGRP neurons in PB (Mumphrey et al., 2016) and this may also be the case in the present study. RYGB is associated with an increased postprandial response of PYY(3-36) and GLP-1 in humans (Le Roux et al., 2006; Dirksen et al., 2013) and in rats exposed to ileal transposition (Strader et al., 2005). The increase in PYY(3-36) and GLP-1 may contribute to c-fos reactivity induced in the NTS to PB to CeA pathway demonstrated by Mumphrey and colleagues (Mumphrey et al., 2016) as well as in the present study. The CGRP neurons in the PB can be activated by different pathways of which the most studied are by an reduction of the tonus of the hypothalamic NPY/AgRP neurons (Wu et al., 2009) as well as increased POMC/CART activity and by glutamatergic innervation from hindbrain (van der Kooy & Koda, 1983). For the synergistic effect of PYY(3-36) and Ex4, all pathways may have contributed to the increased activation of neurons in the PB. We also observed significant c-fos reactivity in CeA and BST in the combination group. The CGRP neurons are demonstrated to project to CeA and may contribute to this. In BST the enhanced c-fos may also be caused by a reduced tonus of the NPY/AgRP neurons (Betley et al., 2013) together with increased input from CeA (Carter et al., 2013). The c-fos activated pathways in the present study support previous studies showing that appetite reduction involves AP/NTS to PB to CeA neuronal circuits.

Interestingly, activation of brain regions such as LRNm and SUT was novel and specific for the combination group. To our knowledge, LRNm and SUT have not been described to be associated with regulation of energy balance, but appear to be involved in jaw movements which could be related to the initiation of eating (Fujio et al., 2016; Alstermark & Ekerot, 2013). Furthermore, according to Allen brain atlas the brain area SUT sends neuronal projections to PB in normal C57BL/6 J mice, indicating a potential interaction between PB and SUT which may be induced by PYY(3-36) and Ex4 in combination. The LRNm appears to regulate several functions as it provides the cerebellum with signals to control posture, reaching, grasping, locomotion, scratching and respiration (Alstermark & Ekerot, 2013; Xu et al., 2013), involvement in pain (Ossipov & Gebhart, 1986) and regulation of heart rate and blood pressure in cats (Thomas et al., 1977). A viral-genetic tracing study has also demonstrated that LRNm project to locus ceruleus (LC) (Schwarz et al., 2015) a brain region known to be involved in arousal and attention and recently also in reward processing in monkeys (Bouret & Richmond, 2015). In the present study, increased c-fos reactivity was observed in LC, but this was also observed with Ex4. Thus, the observed enhancement of c-fos response in SUT and LRNm needs further investigation to understand if the regions contributed to the additive/synergistic effect of PYY(3-36) and Ex4 on food intake.

In conclusion, the present study demonstrated global brain c-fos reactivity in mice treated with PYY(3-36) and Ex4 in combination. The additive reduction in food intake by PYY(3-36) and Ex4 was associated with synergistic enhanced c-fos reactivity in the rostral part of the mediobasal ARH and the appetite-reducing AP/NTS to PB to CeA pathway. The combination of PYY(3-36) and Ex4 also activated LRNm and SUT which has not previously been reported in the regulation of energy homeostasis. In addition to appetite reduction, the significant increase in c-fos reactivity may also contribute to regulation of other biological responses that are not investigated in the present study. As PYY(3-36) and Ex4 act on different neurons leading to recruitment of different signalling pathways within and to the brain, an interaction of these pathways may contribute to their additive/synergistic action. Thus, PYY(3-36) boosts the effect of Ex4 possibly by inducing less inhibition of neuronal activity leading to an enhanced neuronal activity induced by Ex4.

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Author contributions

Marina Kjaergaard has been responsible for the design, experimental work, data processing, biological interpretation of results, and for writing the manuscript. Casper Jensen has been involved in the whole brain c-fos data processing. Birgitte S. Wulff, Anna Secher and Kirsten Raun have been involved in the design of studies, the biological interpretation of the results and critical comments to the manuscript. Jens F. Rehfeld has been a part of the project from the start and has made critical comments to the final manuscript.

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Declaration of interest

Novo Nordisk markets liraglutide for the treatment of diabetes and obesity, and semaglutide for diabetes. Semaglutide is furthermore in development for obesity. Birgitte S. Wulff, Anna Secher, Casper Bo Gravesen Salinas and Kirsten Raun are full time employees at Novo Nordisk and stock owners through an employee offering program.

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